JOURNAL OF BACTERIOLOGY, Mar. 1977, p. 1234–1238
Vol. 129, No. 3
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Oscillations in the Synthesis of Cell Wall Components in Synchronized Cultures of *Escherichia coli*

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Received for publication 20 October 1976

The rate of cell wall synthesis with respect to both proteins and lipids was determined in synchronized cultures of *Escherichia coli* B/r. Whereas the rate of total protein synthesis showed an exponential increase with cell age, the rate of incorporation of proteins and lipids into cell wall had a maximum at a cell age of 30 to 35 min, 15 min before cell division. This oscillation was observed in both the cytoplasmic membrane and in the outer membrane of the cell envelope.

The morphological manifestation of bacterial cell division starts with formation of a septum at the presumptive equatorial division site. This process must be strictly controlled in the time and location of the newly synthesized cell wall material. The cytoplasmic membrane is believed thereby to play an essential role as mediator of information, coupling chromosome replication and segregation to the formation of new polar caps (11). Attempts have been made to correlate the morphological changes with the synthesis of cell wall components, in relation to either cellular location or cell age (10, 15–17, 20, 22).

A defined incorporation zone has been found for specific precursors of murein (22) and for the λ-receptor, a protein of the outer membrane of *Escherichia coli* (20). The failure to detect localized synthesis of lipid material might be caused by the use of techniques that interfere with the fluidity of the membranes (15). An age-dependent activity of certain cell wall components has indeed been found in a few studies: Ohki (17) could show a stepwise increase in the amount of cytochrome *b1* and the transport of 1-α-glycerophosphate 15 to 25 min before cell division at a generation time of 100 to 110 min. Also, murein synthesis (10) and incorporation of lipoprotein into the outer membrane of *E. coli* (12) occur at the maximal rate during the time of septation. In *Bacillus subtilis*, proteins of the membrane are synthesized more rapidly in the latter part of the division cycle (21). Phospholipid synthesis in *B. subtilis* was described to be continuous (21), in contrast to *E. coli* (17). The difference in synchronization techniques and organisms examined makes it somewhat difficult to compare these results, although they point to a general activated cell wall metabolism during the time of septum formation.

In this communication, we describe the rate of synthesis of proteins and lipids in whole cell walls and in separated membranes in synchronized *E. coli* B/r.

MATERIALS AND METHODS

Synchronization of *E. coli* WM 231. *E. coli* WM 231, a T1-resistant derivative of *E. coli* B/r (ATCC 12407), was synchronized by a modification of the membrane selection technique of Helmstetter and Cummings (9) described by Messer (14). Cells were grown in glucose minimal medium (8) containing: 2 g of NH_4Cl; 6 g of $Na_2HPO_4 \cdot 2H_2O$; 3 g of KH_2PO_4; 3 g of NaCl; 0.175 g of $MgSO_4 \cdot 7H_2O$; and 2 g of glucose in 1 liter of demineralized water. Cell number and cell volume were determined with a Coulter counter combined with a Nuclear Data multichannel analyzer.

Radioactive labeling of cells. Five to 10 ml of the synchronized culture was pulse-labeled for 3 min with either [^{3}H]acetate (Radiochemical Centre, Amersham, England; 250 mCi/mmol) or radioactive leucine ([^{3}H]leucine, 5 mCi/mmol; [^{14}C]leucine, 348 mCi/mmol; Radiochemical Centre, Amersham, England). For specific activity in the growth medium, see legends to figures. Incorporation of radioactive material was stopped by freezing the pulse-labeled cultures in liquid nitrogen. Lipid extraction was performed as described by Ames (1). Incorporation into acid-precipitable material was determined by filtration of 5% trichloroacetic acid-treated samples onto membrane filters (Sartorius, Göttingen, Germany; 0.45-μm pore size). The dried filters were counted in scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene in a Nuclear Chicago Isocap 300 scintillation counter.

Fractionation of cells into cytoplasm and cell walls. Frozen cells were quickly thawed in warm water, and fractionation continued at 4°C. After centrifugation at 27,000 × *g* (Sorvall centrifuge), cells were resuspended in 100 μl of 0.75 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris)-

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JOURNAL OF BACTERIOLOGY, Mar. 1977, p. 1239–1244
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Vol. 129, No. 3
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Activity of Murein Hydrolases in Synchronized Cultures of *Escherichia coli*

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Received for publication 20 October 1976

Murein hydrolase activities were analyzed in synchronized cultures of *Escherichia coli* B/r. Cell wall-bound murein hydrolase activities, including the penicillin-sensitive endopeptidase, increased discontinuously during the cell cycle and showed maximum activity at a cell age of 30 to 35 min (generation time, 43 min). Maximum activity was observed at the same time that the rate of cell wall synthesis reached its maximum. These oscillations depended on the termination of replication: no increase in hydrolase activity was found if deoxyribonucleic acid synthesis was inhibited at an early time in the life cycle. In contrast, the activity of another murein hydrolase that was not tightly bound to the membrane (transglycosylase) increased exponentially with time, even when deoxyribonucleic acid synthesis was inhibited.

The process of bacterial cell division is directly coupled with deoxyribonucleic acid (DNA) replication. Only if a chromosomal replication cycle has been completed can subsequent cell division take place (3, 11, 15, 18). The end of a replication round at the same time is a signal that triggers a sequence of events necessary for cell separation. The first such event is an increased incorporation of murein-specific precursors into the cell and a simultaneously increased penicillin sensitivity, which indirectly reveals a higher activity of murein-hydrolyzing enzymes at that cell age (12).

Septum formation requires a defined topological alteration of the cell wall at a defined time. The morphological changes the cell must go through during the life cycle are reflected by the shape of the murein sacculus, which is a covalently closed macromolecule surrounding the cell (25). Its modification is believed to occur via a highly coordinated collaboration between murein-synthesizing and -hydrolyzing enzymes (4, 23, 25).

In *Escherichia coli* several murein hydrolase activities are known, most of which seem to be membrane bound (4, 7, 21). To gain more information about the regulation of murein metabolism in the cell cycle, we measured the activity of various murein hydrolases that differ in specificity as well as in their localization within the cell. In addition, the dependence of murein hydrolase activities on DNA replication was analyzed.

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MATERIALS AND METHODS

Synchronization of *E. coli* WM 231. *E. coli* WM 231, a T1-resistant derivative of *E. coli* B/r (ATCC 12407), was synchronized by a modification of the membrane selection technique of Helmstetter and Cummings (10) described by Messer (19). Cells were grown in glucose minimal medium (9). Cell number and cell volume were determined with a Coulter counter combined with a Nuclear Data multichannel analyzer.

Fractionation of cells in cytoplasm and cell wall. The fractionation procedure was carried out as described in the preceding paper (6). The volume of cytoplasmic fractions was 300 μl; the cell wall sediments were resuspended in a final volume of 150 to 200 μl. Protein was determined as described by Lowry et al. (17).

Determination of murein hydrolase activities. Total cell wall-bound murein hydrolases were tested with [^{3}H]DAP (*meso*-α,ε-diaminopimelic acid)-labeled sacculi as a substrate (8). Per test, 10 μl of sacculi (0.5 mg of murein per ml, 10^6 cpm/ml) and 30 μl of the cell wall fractions (5 to 15 μg of protein) were incubated in 100 μl of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, 0.01 M Mg^{2+}, and 1% (wt/vol) Triton X-100, pH 6.2, for 2 h at 37°C. The enzyme reaction was stopped by cooling to 0°C. After addition of serum albumin (10 μl; 5% solution) and 10 μl of 50% trichloroacetic acid, samples were centrifuged for 2 min at 12,000 × *g*, and radioactivity in 90 μl of supernatant plus 0.5 ml of 0.1 M NaOH was counted in 5 ml of Instagel scintillation fluid (Packard, Zürich, Switzerland).

Endopeptidase activity was measured with [^{3}H]DAP-labeled muropeptide C3 (25) as a substrate {50 μl of cell wall suspension in 0.01 M Tris-maleate buffer, 0.01 M Mg^{2+}, 1% Triton X-100 (wt/vol), pH 7.8, containing 20 nmol of C3 (=1.5 × 10^5 [^{3}H]DAP

cpm}. Incubation was carried out for 2 to 3 h at 37°C, and the reaction was stopped in a boiling-water bath. After centrifugation for 2 min (12,000 × *g*), 40 μl of the supernatant was lyophilized and suspended in 5 μl of distilled water, and the dimeric substrate was separated from monomeric product by paper chromatography (16). The chromatogram was cut into strips of 4 mm, and radioactivity was counted in 5 ml of toluene scintillation fluid (5 g of 2,5-diphenyloxazole in 1 liter of toluene).

Activity of the transglycosylase (13) was determined as follows (E. W. Goodell and U. Schwarz, manuscript in preparation): cells were centrifuged (27,000 × *g*, Sorvall centrifuge), resuspended in 100 μl of 0.01 M Tris-hydrochloride buffer, 5 mM ethylenediaminetetraacetic acid (EDTA), and 2% Triton X-100 (wt/vol), pH 7.8 (cell concentration now 4 × 10^{10} to 1 × 10^{11} cells/ml), and incubated for 30 min at 30°C. After centrifugation at 48,000 × *g* for 30 min (Sorvall centrifuge), 20 μl of the supernatant was taken as enzyme source. The reaction mixture (100 μl [total volume] of 0.01 M Tris-maleate buffer-0.01 M Mg^{2+}, pH 6.0) containing the enzyme and [^{3}H]DAP-labeled sacculi as substrate (see above) was incubated for 60 to 90 min at 37°C. Determination of radioactive trichloroacetic acid-soluble reaction products was done as described above. The reaction products, as checked by paper chromatography, were the same as those reported for purified transglycosylase (13) and different from those observed with cell walls as an enzyme source (R. Hakenbeck, Ph.D. thesis, Freie Universität Berlin, 1976).

RESULTS

Activity of murein hydrolases. In *E. coli*, a set of different murein-hydrolyzing enzymes has been described (4, 25). They differ not only in their substrate specificity within the murein network (sugar chains or peptide moieties) but also with respect to their location within the cell (cell wall or cytoplasm). A transglycosylase has been purified from soluble extracts (8, 13). All other activities analyzed so far are tightly bound to the cell envelope (for review see reference 4). A penicillin-sensitive endopeptidase is located exclusively in the cytoplasmic membrane (5). This enzyme is probably identical to the one designated carboxypeptidase I (2, 5).

With cell wall fractions as the enzyme source, we determined the activity of total cell wall-bound hydrolases (including lysozyme, amidase, and endopeptidase) by measuring the liberation of trichloroacetic acid-soluble products from [^{3}H]DAP-labeled sacculi as a substrate. Endopeptidase activity was determined by using the conversion of the dimeric subunit of the murein C3, obtained after lysozyme digestion, into monomers (25). The transglycosylase could be extracted from cells and thereby separated from all other murein-hydrolyzing activities with Triton plus EDTA. Its activity was tested with sacculi as a substrate, again measuring the liberation of acid-soluble product.

These systems allow us to discriminate between the following possible regulation mechanisms for murein-degrading enzymes: (i) all enzymes that attack the murein are regulated in a coordinated way; (ii) differences in regulation exist because of different specificities of the enzymes toward the murein; and (iii) the regulation is correlated with the compartmentalization of the hydrolases, and therefore envelope-bound enzymes, which are in close contact with their substrate, are regulated together and differently from "soluble" enzymes.

In Fig. 1 the activities of total cell wall-bound murein hydrolases are shown in synchronized *E. coli* B/r. Cell samples were taken at the indicated times, and fractionation of the cells and enzyme tests were performed as described in the legend and in Materials and Methods. Since only a limited amount of synchronized cells can be obtained per experiment, two experiments are shown; in Fig. 1a samples are taken through approximately two cell cycles, whereas in Fig. 1b samples are taken more frequently within one cell cycle. In Fig. 2 the activity of the endopeptidase in synchronized cells is shown. Figure 3 shows the activity of transglycosylase at different cell ages.

The soluble, or only loosely membrane-bound, transglycosylase and cell wall-bound enzymes clearly behaved differently in the cell cycle. The activity of total cell wall-bound murein hydrolases and of the cytoplasmic membrane-bound endopeptidase increased discontinuously, with maximum enzyme activity at a cell age of about 30 min, 15 min before cell division. This pattern was observed again in the second cell cycle. On the other hand, the activity of the transglycosylase showed a continuous exponential increase with cell age.

Although cell wall-bound enzyme and endopeptidase activities are distributed differently in the outer and cytoplasmic membranes (5), the pattern of their activities versus cell age was identical. Endopeptidase was found nearly exclusively in the cytoplasmic membrane; the other membrane-bound murein hydrolases were located both in the outer and in the cytoplasmic membrane. It could therefore well be that the cytoplasmic membrane plays the essential role as a transmitter of information and is thus responsible for the regulation of cell wall-bound murein hydrolases. This point will be discussed in detail later.

The time when maximal murein hydrolase activity was observed corresponded with the indirect determination of increased autolytic

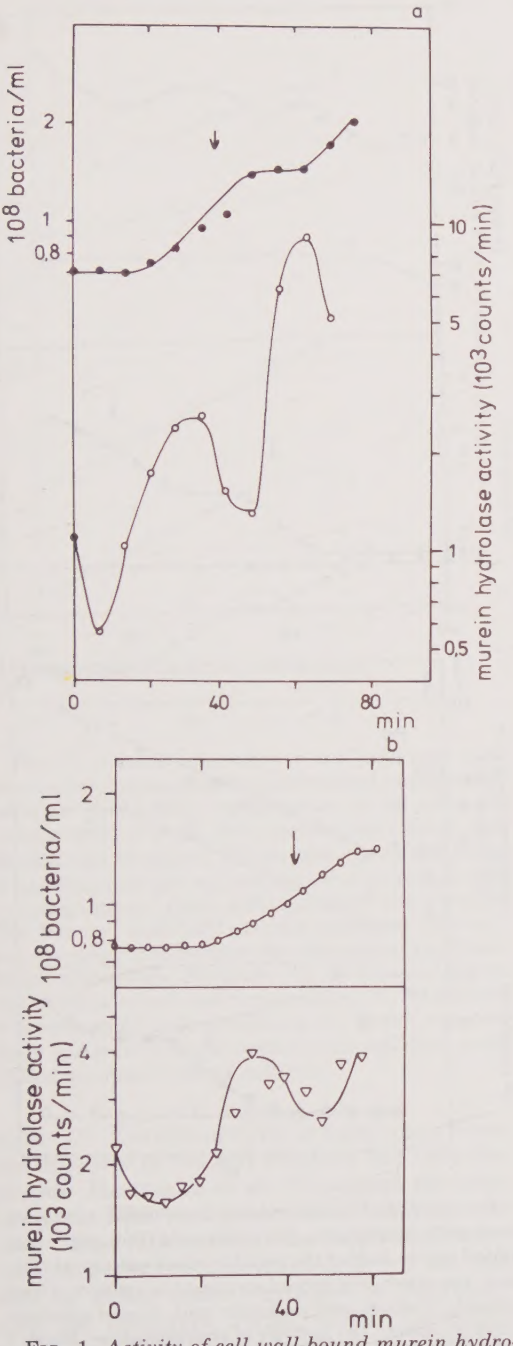

FIG. 1. *Activity of cell wall-bound murein hydrolases in synchronized cells. (a) 10-ml samples were taken every 7 min, and 30 μl of the prepared cell wall fraction (total volume, 200 μl) was taken as the enzyme source. Incubation with sacculi as substrate was carried out for 3 h. (b) 8-ml samples were taken every 4 min, and 40 μl of 150-μl cell wall suspension was tested (2-h incubation) for enzyme activity. Arrows indicate the inflection points of the growth curves in all figures.*

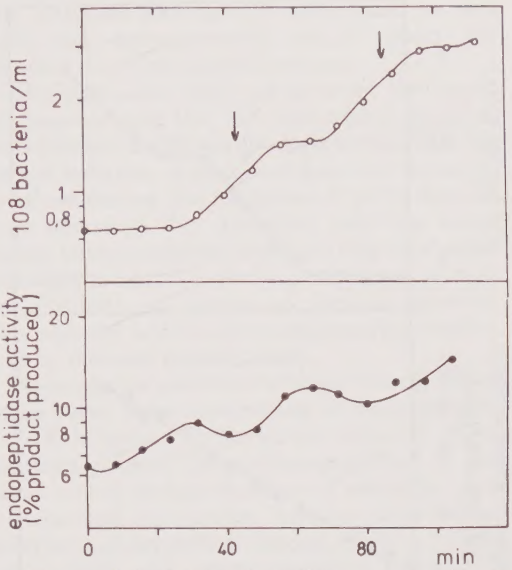

FIG. 2. *Activity of endopeptidase versus cell age. Every 8 min, 8-ml samples were taken and cell wall fractions were prepared (150-μl total volume). Endopeptidase activity was tested with 30 μl of cell wall suspension and [^{3}H]DAP-labeled C3 as a substrate. Incubation time was 2.5 h. Symbols: (○) Cell number; (●) endopeptidase activity.*

activity as measured by penicillin sensitivity of synchronized cells (10).

Murein hydrolase activity and the DNA replication cycle. A difference in behavior between transglycosylase and the other, membrane-bound, murein hydrolases was also observed when we tested the dependence of their activity on the termination of DNA replication.

One-half of a synchronized culture was incubated with nalidixic acid starting at cell age t = 0; the other part served as a control culture. Endopeptidase and transglycosylase activities were determined in independent experiments (Fig. 4a and b, respectively). Cell samples were taken at the indicated times, and enzyme tests were performed as described before. Only the endopeptidase activity was affected by the inhibition of DNA replication; after 15 to 20 min no further increase in activity was observed. The activity of the transglycosylase, in contrast, continued to increase as in the control culture up to at least 60 min.

The total amount of cell wall protein was also affected in a culture treated with nalidixic acid (Fig. 5). The amount of protein in the cell wall increased monotoneously, but only by a factor of 1.4 per generation. The amount of protein in the cytoplasm, on the other hand, increased at the same rate as in the control culture.

During treatment with nalidixic acid, the

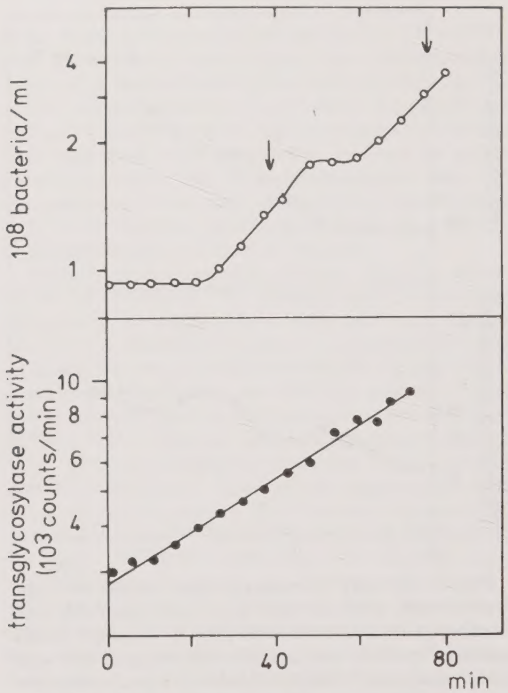

FIG. 3. *Activity of transglycosylase in synchronized cells. Duplicate 4-ml samples were taken every 6 min. Cells were extracted with EDTA and Triton X-100, and the activity of the transglycosylase was determined with 40 μl of the solubilized material (total volume, 150 μl), with [³H]DAP-labeled sacculi as a substrate. Incubation time 80 min.*

cells did not divide but only grew in length. Assuming that the length of a "young" cell equals the radius of the cross section, one doubling leads to an enlargement of the surface by a factor of 1.33. This value agrees with the observed increase in membrane material in the presence of the drug.

DISCUSSION

In the cell cycle of *E. coli* B/r, cell age-dependent murein hydrolase activity was only observed for cell wall-bound enzymes and not for the soluble, or less tightly bound, transglycosylase. The maximum activity for membrane-bound murein hydrolases, including the penicillin-sensitive endopeptidase, occurred at 30 to 35 min in the first cell cycle and at a corresponding time, 15 min before cell division, in the second cell cycle. No differences were found for enzymes that are located in both layers of the cell envelope and between the endopeptidase that is specifically bound to the cytoplasmic membrane.

In *E. coli*, no murein hydrolytic activity can be observed when intact cells are incubated in

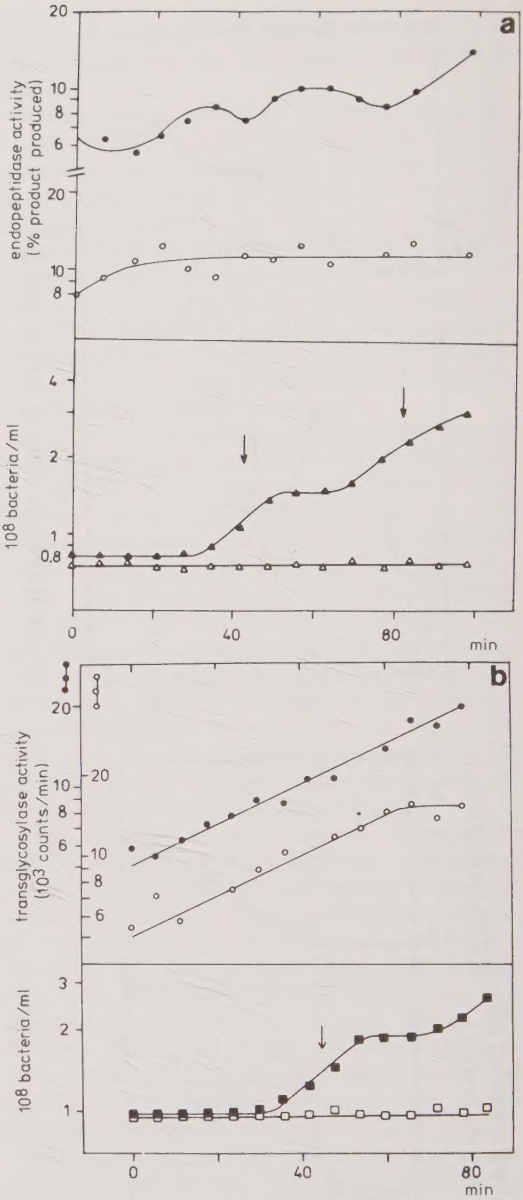

FIG. 4. *Effect of the inhibition of DNA synthesis on murein hydrolases. Nalidixic acid (10 μg/ml) was added to one-half of the synchronized culture at time zero; the other part served as a control culture. Open symbols: Culture with nalidixic acid. Closed symbols: Control culture. (a) Activity of endopeptidase. Every 7 min, 8-ml samples were taken and cell wall fractions (200 μl) were prepared. Endopeptidase activity was tested with 30 μl of cell wall fraction and [³H]DAP-labeled C3 as substrate; incubation time was 2.5 h. (b) Activity of transglycosylase. Every 6 min, 4-ml samples were taken. After preparation of the Triton-EDTA-solubilized material (100 μl, final volume), activity of the transglycosylase was determined with 25 μl of solubilized material and [³H]DAP-labeled sacculi as substrate.*

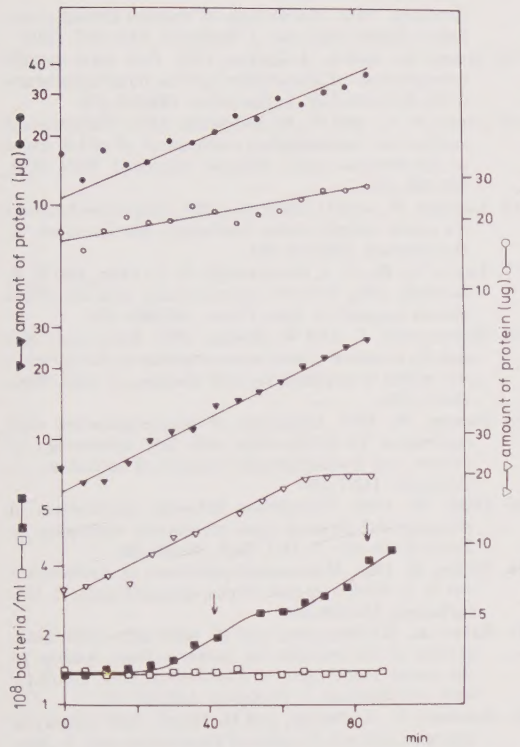

FIG. 5. *Amount of protein in cell wall and cytoplasm after treatment of a synchronized culture with nalidixic acid. After resuspension of the collected "young" cells (t = 0), the synchronized culture was divided into two parts. To one part was added 10 µg of nalidixic acid per ml, and the other parts served as a control culture. Cells (8-ml samples) were fractionated into cell wall (200 µl) and cytoplasm (300 µl), and the amount of protein was determined in 50 and 20 µl, respectively. Symbols:* (○, ●) *Amount of protein in 50 µl of cell wall preparation;* (▽, ▼) *amount of protein in 20 µl of cytoplasm;* (□, ■) *cell number. Open symbols: Culture treated with nalidixic acid. Closed symbols: Control culture.*

buffer (7). Enzyme activity is found only when the integrity of the cell envelope has been destroyed. Hartmann et al. (7) suggest the existence of a "barrier," preventing these enzymes from uncontrolled action. Our technique for determining enzyme activities destroys the cell wall by either sonic treatment or treatment with the detergent Triton X-100. Therefore, such a topological control system may not become obvious in our experiments. We think that we probably measure the total amount of enzyme present in the cell. The decrease of the cell wall-bound murein hydrolase activity during the division time may point to a high turnover rate for the enzymes. However, it is also possible that extraction of the enzymes with Triton does not dissociate complexes between the enzymes and specific inhibitors. In this case, our measurements would reflect the amount of active enzyme present.

The difference observed between the transglycosylase and the cell wall-bound enzymes could reflect a different biological role. The different behavior of endopeptidase and transglycosylase during the inhibition of DNA replication points in this direction: only the membrane-bound endopeptidase activity (and other membrane-bound hydrolases; data not shown) is affected and shows no further increase, whereas the activity of transglycosylase continues to increase exponentially.

Recently the activities of three murein hydrolases have been determined in toluenized *E. coli* K-12 cells of different cell ages (1). These authors showed that carboxypeptidase II was most active during the time of septation, consistent with our results. However, they did not find an age-dependent change in the activities of amidase and carboxypeptidase I, which is probably identical to the endopeptidase tested by us (2, 5). We do not know whether this discrepancy is due to the different preparations of the enzyme source or to the different enzyme activities used to test the enzyme system (carboxypeptidase activity versus endopeptidase activity).

Murein hydrolases have been proposed to have different functions in cell elongation and septum formation (23), arguing that the process of cell growth could be divided into longitudinal and equatorial synthesis of cell wall material. The specificity of the transglycosylase (13) suggests that it might be involved in the kind of enlargement (reshuffling) of the sacculus that must take place at any cell age (24), whereas the formation of the cross wall might need different or enhanced action of other murein-degrading enzymes.

Different functions of the *E. coli* envelope show maximum activity at the time when the septum is formed: the activity of membrane-bound murein hydrolases, the rates of overall synthesis of membrane proteins and lipids (6), and the synthesis and/or incorporation of specific proteins (cytochrome *b1* [20]; lipoprotein [14]; λ-receptor [22]). This suggests that equatorial synthesis of cell wall is coordinately controlled in the sense that a common trigger that is dependent on the termination of DNA replication induces the synthesis of all or most components required for septum formation.

ACKNOWLEDGMENTS

We thank J. Reeve, U. Schwarz, and T. A. Trautner for many stimulating discussions.

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hydrochloride buffer, pH 7.8. A 200-μl amount of 1.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, was added. Cells were sonically treated for 3 min (MSE ultrasonic disintegrator, model 100 W), and cell wall fractions were sedimented at 48,000 × *g* for 1 h (Sorvall centrifuge). The supernatant was taken as the cytoplasmic fraction, and the sediment (cell walls) was resuspended in 150 to 200 μl of Tris-maleate buffer (0.01 M, pH 6.1). Protein determination was carried out as described by Lowry et al. (13).

Separation of the cell wall into cytoplasmic and outer membranes. The fractionation procedure described by Osborn et al. (19), based on spheroplast formation by lysozyme treatment, cannot be used with frozen cell cultures. Therefore we used a modification of that method (5) in which separation of the two membrane layers is achieved by trypsin treatment (3) of sonically treated cell walls. The membranes were fractionated in a stepwise sucrose gradient (25 to 55%, wt/vol) in an SW41 rotor at 38,000 rpm for 13 to 14 h in a Spinco ultracentrifuge model L2-65B (19). Fractions (0.3 to 0.4 ml each) were collected from the bottom of the gradient.

RESULTS

In most of the experiments described, cell envelope and cytoplasm were fractionated after ultrasonic disintegration of cell samples taken at different times (cell age) from synchronized cultures. It was important to ensure that no superimposed irregularity was introduced by that technique, since Clark (4) showed a cell age-, i.e., size-, dependent differential killing of synchronized cells by ultrasound. Therefore the cells were pretreated in sucrose-Tris-EDTA buffer (see Materials and Methods), which destabilizes the cell wall. After disintegration, fewer than 10^{-5} cells survived (as tested by the ability to form colonies). No dependence of cell fragility on cell age was observed under these conditions.

Cell fractions were prepared by one centrifugation step after the ultrasonic treatment; the total amount of protein in the resulting pellet (cell wall fraction) and in the supernatant (cytoplasmic fraction) increased exponentially with cell age (Fig. 1). This result further substantiates the reliability of the fractionation method.

The rate of lipid synthesis was determined by using acetate as a precursor for the fatty acid moieties of phospholipids (23). To demonstrate that acetate is not metabolized into other compounds during the pulse, we determined the radioactivity extractable by chloroform/methanol (1) after a 3-min pulse, given to a exponentially growing culture, in both cell wall and cytoplasmic fractions. In the cell wall, radioactivity was found exclusively in the organic phase, indicating that all acetate label is incorporated into lipids. This enabled us to determine the lipid in cell walls as trichloroacetic acid-precipitable material. In the cytoplasmic fraction about 50% of the label appeared in the aqueous phase, obviously due to precursors in fatty acid synthesis as well as to the cellular pool of acetate.

Rate of cell wall synthesis. Synchronized cells were pulse-labeled for different lengths at different times in the cell cycle with [^{3}H]acetate and [^{14}C]leucine. The radioactive precursors were taken up by the cells with the same kinetics, irrespective of cell age (Fig. 2), indicating that there were no gross differences in pool size

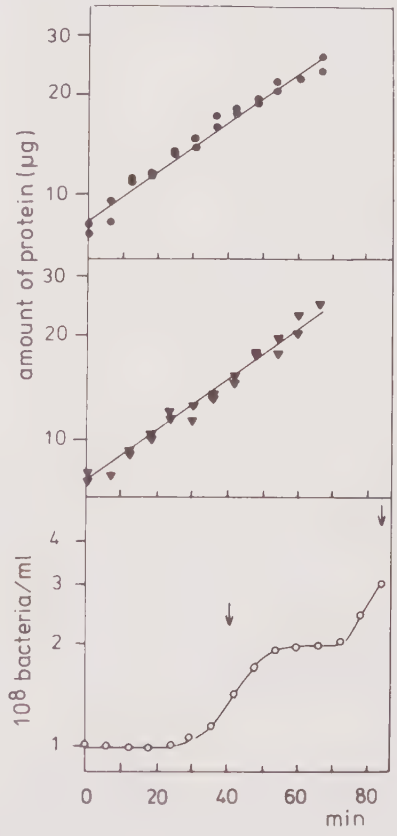

FIG. 1. *Amount of protein in cytoplasmic and cell wall fractions. Every 6 min, duplicate samples (5 ml) of the synchronized culture were frozen in liquid nitrogen. After preparation of cell fractions (see Materials and Methods), the amount of protein in the cytoplasm (30 μl of a total 225 μl) and in cell wall fractions (60 μl of a total 150 μl) was determined by the technique of Lowry et al. (13). Arrows in all figures indicate the inflection points of the growth curves, i.e., the time of division for an average cell. Symbols: (●) Amount of protein in cytoplasmic fractions; (▲) amount of protein in cell wall fractions; (○) cell number.*

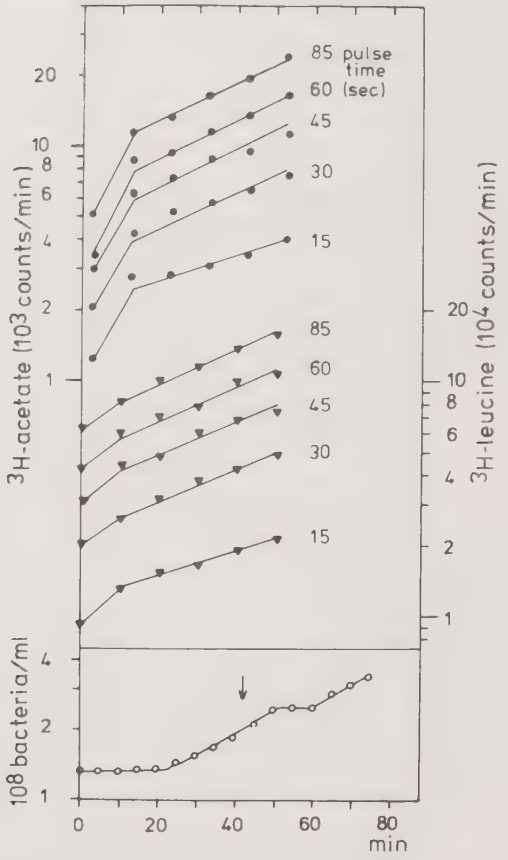

FIG. 2. *Incorporation kinetics of [^{3}H]acetate and [^{3}H]leucine in synchronized cells. At different times during the growth cycle, 5 ml of a synchronized culture was labeled with either 90 μCi of [^{3}H]acetate (3.2 mCi/mg) or 9 μCi of [^{3}H]leucine (9 mCi/mg) per ml. After 15, 30, 45, 60, and 85 s, 1-ml aliquots were taken and 5 ml of cold trichloroacetic acid (5%) was added. Samples were filtered onto membrane filters, and radioactivity was determined. Symbols: (●) [^{3}H]acetate counts per minute; (▲) [^{3}H]leucine counts per minute; (○) cell number.*

at different cell ages. The reduced rate of uptake of acetate and leucine at cell age $t = 0$ was due to the temperature shock imposed on the cells during synchronization (this is required when many cells are needed in the experiment; 14).

When such a temperature shock was avoided, the incorporation of both [^{3}H]acetate and [^{14}C]leucine into whole cells increased exponentially throughout the cell cycle (Fig. 3).

In contrast, the incorporation of these precursors into cell wall material did not increase monotoneously (Fig. 4). The rate of cell wall synthesis was minimal at a cell age of approximately 10 min (between 8 and 12 min in individual experiments) and then increased until a maximum was reached approximately 15 min before cell division (cell age between 27 and 36 min; mean, 30 min), i.e., at the same time when polar cap formation started. The rate of cell wall synthesis then decreased again during cell division and reached a minimum corresponding to the one observed in the first cycle shortly after division. Another maximum in the rate of synthesis was reached at approximately 74 min (60 to 80 min), again about 15 min before cell division.

Rate of synthesis of outer and inner membranes. The cytoplasmic and outer membranes of the *E. coli* envelope showed distinct functions. Synthesizing activities are probably only found in the cytoplasmic membrane, for ex-

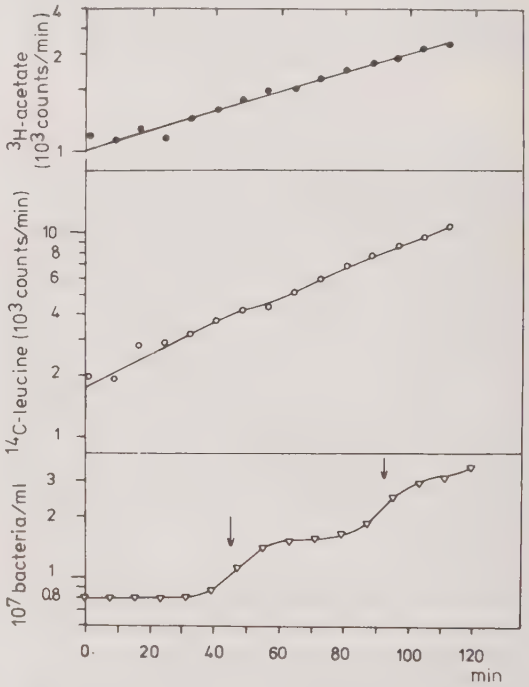

FIG. 3. *Incorporation of [^{14}C]leucine and [^{3}H]-acetate during a 3-min pulse into unfractionated synchronized cells. Synchronized growth in this experiment was started by collecting "young" cells from the membrane for 3 min in a prewarmed flask. Incubation was started immediately. Every 8 min, duplicate samples of 2.5 ml were added to 0.5 ml of radioactive medium (95 μCi of [^{3}H]acetate per ml, 3.2 mCi/mg; 5 μCi of [^{14}C]leucine per ml, 5 mCi/mg, final concentrations). After 3 min, incorporation was stopped by freezing in liquid nitrogen. The cell suspension was thawed, and nonradioactive carrier cells were added. A 150-μl amount of this suspension was precipitated with trichloroacetic acid, and the radioactivity was determined. Points are the mean values of the duplicate samples.*

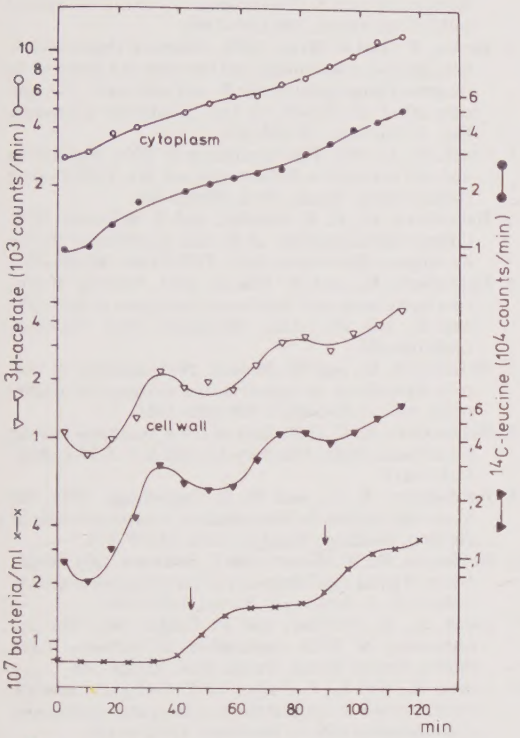

FIG. 4. *Rate of incorporation of acetate and leucine into cytoplasm and cell wall of a synchronized culture. Cells were synchronized and pulse-labeled as described in the legend to Fig. 3. After fractionation into cytoplasm and cell wall, trichloroacetic acid-precipitable radioactivity was determined in all cell fractions. Symbols: (○) [^{3}H]acetate and (●) [^{14}C]leucine in cytoplasmic fractions; (▽) [^{3}H]acetate and (▼) [^{14}C]leucine in cell wall fractions; (×) cell number.*

ample the enzymes for the synthesis of lipopolysaccharides, which are then transported and incorporated into the outer membrane (18). Membrane material can be transported and incorporated into the outer membrane matrix by at least two different mechanisms: sites of adhesion between both membranes seem to be the sluice for lipopolysaccharide (16), whereas the λ-receptor protein is synthesized late in the cell cycle and incorporated only into the presumptive division zone (20). It might be possible that in a pulse of 3 min, as used in the experiments described above, unequal proportions of the two membrane components were labeled and incorporated.

Synchronized cells were pulse-labeled with [^{3}H]acetate at various times. Nonradioactive carrier cells were added, and the cytoplasmic and outer membranes were separated (Fig. 5a).

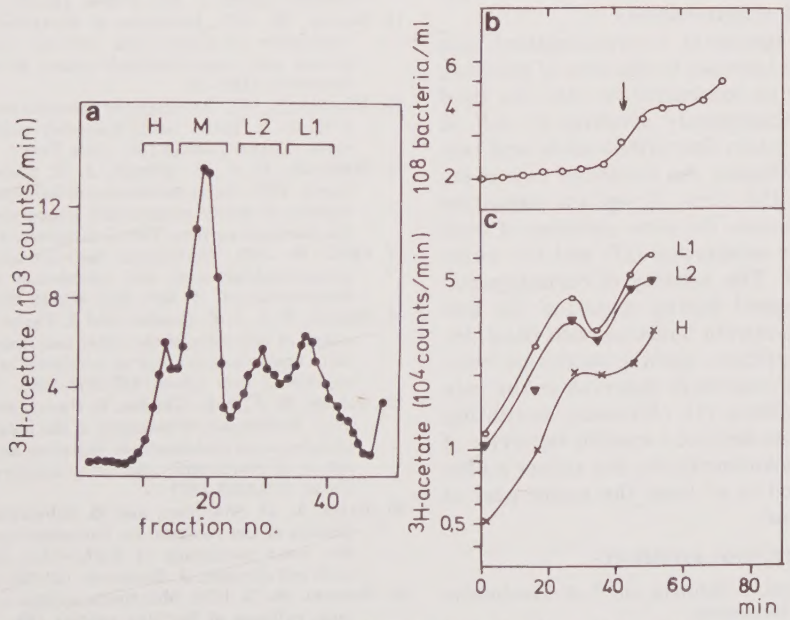

FIG. 5. *Rate of [^{3}H]acetate incorporation into the membrane fractions of the cell envelope during synchronous growth. At the indicated times, 10 ml of a synchronized culture was pulse-labeled for 3 min with [^{3}H]acetate (50 μCi/ml; 3.2 mCi/mg). Preparation of cell walls and separation into cytoplasmic and outer membranes were as described in Materials and Methods. (a) Separation of cell wall in a sucrose gradient. (b) Cell number of a synchronized culture. (c) Incorporation of [^{3}H]acetate into the outer membrane (H) and the cytoplasmic membrane (L1 and L2). Each point represents the corresponding integrated peak from gradients of the kind shown in (a).*

In Fig. 5 the rate of lipid incorporation into H-band (outer membrane) and L-bands (cytoplasmic membrane) is shown at different cell ages. The oscillation in the rate of incorporation of acetate was more prominent in the cytoplasmic membranes than in the H-band. The H-band in this technique is contaminated by about 10% L-band material (5). If a correction is done for this contamination, the oscillation in the H-band becomes even more evident, and the maximum and minimum rates of acetate incorporation occur 5 to 10 min later than in the L-band. This suggests that in both membrane layers the rate of lipid incorporation oscillates with cell age.

DISCUSSION

The rate of cell wall synthesis showed an oscillating increase during the cell cycle. This held for fatty acid (i.e., lipid) as well as protein incorporation into cell wall material. A maximum was observed during the time of cell septation, under our conditions (generation time of 43 min) approximately 15 min before cell division. Both the cytoplasmic and the outer membranes of the *E. coli* envelope showed this oscillation.

The total amount of cell wall protein showed an exponential increase because the small deviations from an exponential increase obtained from an integration of the rate curves were below the level of detectability.

O. Pierucci (personal communication) also found a sudden increase in the rate of glycerol, acetate, or serine incorporation into the lipid fraction of synchronously dividing *E. coli* at about the time when deoxyribonucleic acid replication was initiated. An enhanced rate of incorporation at the time of septum formation (12) has been shown for some proteins of both the cytoplasmic membrane (17) and the outer membrane (12). The activity of carboxypeptidase II is increased during septation (2), and cell wall-bound murein hydrolase activities, including endopeptidase, show a maximum coincident with the maximum observed in the rate of cell wall synthesis (7). Obviously oscillating rates of synthesis are not a specific behavior of certain proteins and/or lipids, but rather a phenomenon related to at least the major part of cell wall material.

ACKNOWLEDGMENTS

We thank J. Reeve, U. Schwarz, and T. A. Trautner for many stimulating discussions.

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